

Amendments to the Specification

Please replace the third full paragraph of page 1 with the following amended paragraph:

Fluorescent molecules are attractive as reporter molecules in many assay systems because of their high sensitivity and ease of quantification. Recently, fluorescent proteins have been the focus of much attention because they can be produced in vivo by biological systems, and can be used to trace intracellular events without the need to be introduced into the cell through microinjection or permeabilization. The green fluorescent protein of *Aequorea victoria* is particularly interesting as a fluorescent protein. A cDNA for the protein has been cloned. (D. C. Prasher et al., "Primary structure of the *Aequorea victoria* green-fluorescent protein," *Gene* (1992) 111:229-33.) Not only can the primary amino acid sequence of the protein be expressed from the cDNA, but the expressed protein can fluoresce. This indicates that the protein can undergo the cyclization and oxidation believed to be necessary for fluorescence. *Aequorea* green fluorescent protein ("GFP") is a stable, proteolysis-resistant single chain of 238 residues and has two absorption maxima at around 395 and 475 nm. The relative amplitudes of these two peaks is sensitive to environmental factors (W. W. Ward. *Bioluminescence and Chemiluminescence* (M. A. DeLuca and W. D. McElroy, eds) Academic Press pp. 235-242 (1981); W. W. Ward & S. H. Bokman *Biochemistry* 21:4535-4540 (1982); W. W. Ward et al. *Photochem. Photobiol.* 35:803-808 (1982)) and illumination history (A. B. Cubitt et al. *Trends Biochem. Sci.* 20:448-455 (1995)), presumably reflecting two or more ground states. Excitation at the primary absorption peak of 395 nm yields an emission maximum at 508 nm with a quantum yield of 0.72-0.85 (O. Shimomura and F. H. Johnson *J. Cell. Comp. Physiol.* 59:223 (1962); J. G. Morin and J. W. Hastings, *J. Cell. Physiol.* 77:313 (1971); H. Morise et al. *Biochemistry* 13:2656 (1974); W. W. Ward *Photochem. Photobiol. Reviews* (Smith, K. C. ed.) 4:1 (1979); A. B. Cubitt et al. *Trends Biochem. Sci.* 20:448-455 (1995); D. C. Prasher *Trends Genet.* 11:320-323 (1995); M. Chalfie *Photochem. Photobiol.* 62:651-656 (1995); W. W. Ward. *Bioluminescence and Chemiluminescence* (M. A. DeLuca and W. D. McElroy, eds) Academic Press pp. 235-242 (1981); W. W. Ward & S. H. Bokman *Biochemistry* 21:4535-4540 (1982); W. W. Ward et al. *Photochem. Photobiol.* 35:803-808 (1982)). The fluorophore results from the autocatalytic

cyclization of the polypeptide backbone between residues Ser⁶⁵ and Gly⁶⁷ and oxidation of the α - β bond of Tyr⁶⁶ (A. B. Cubitt et al. Trends Biochem. Sci. 20:448-455 (1995); C. W. Cody et al. Biochemistry 32:1212-1218 (1993); R. Heim et al. Proc. Natl. Acad. Sci. USA 91:12501-12504 (1994)). Mutation of Ser⁶⁵ to Thr (S65T) simplifies the excitation spectrum to a single peak at 488 nm of enhanced amplitude (R. Heim et al. Nature 373:664-665 (1995)), which no longer gives signs of conformational isomers (A. B. Cubitt et al. Trends Biochem. Sci. 20:448-455 (1995)).

Please replace the first full paragraph of page 3 with the following amended paragraph:

FIGS. 2A-2C. (A) Stereo drawing of the chromophore and residues in the immediate vicinity. Carbon atoms are drawn as open circles, oxygen is filled and nitrogen is shaded. Solvent molecules are shown as isolated filled circles. (B) Portion of the final $2F_o - F_c$ electron density map contoured at 1.0σ , showing the electron density surrounding the chromophore. (C) Schematic diagram showing the first and second spheres of coordination of the chromophore. Hydrogen bonds are shown as dashed lines and have the indicated lengths in Å. Inset: proposed structure of the carbinolamine intermediate that is presumably formed during generation of the chromophore.

Please replace the fourth full paragraph of page 15 with the following amended paragraph:

The term "stringent conditions" refers to a temperature and ionic conditions used in nucleic acid hybridization. Stringent conditions are sequence dependent and are different under different environmental parameters. Generally, stringent conditions are selected to be about 5°C to 20°C . lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe.

Please replace the second full paragraph of page 29 with the following amended paragraph:

In one embodiment recombinant fluorescent proteins can be produced by expression of nucleic acid encoding for the protein in *E. coli*. Aequorea-related fluorescent proteins are best expressed by cells cultured between about 15[]°C. and 30[]°C. but higher temperatures (e.g. 37[]°C.) are possible. After synthesis, these enzymes are stable at higher temperatures (e.g., 37[]°C.) and can be used in assays at those temperatures.

Please replace the first full paragraph of page 24 with the following amended paragraph:

In another embodiment, an amino acid that is close to a second amino acid within about 0.5 nm of the chromophore can, upon substitution, alter the electronic properties of the second amino acid, in turn altering the electronic environment of the [chromophore] chromophore. Table D presents two such amino acids. The amino acids, L220 and V224 are close to E222 and oriented in the same direction in the [] β -pleated sheet.

Please replace the first full paragraph of page 29 with the following amended paragraph:

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Please replace the fourth full paragraph of page 29 with the following amended paragraph:

Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, transcription enhancer elements, transcription terminators, etc. may be used in the expression vector (see, e.g., Bitter, et al., *Methods in Enzymology* 153:516-544, 1987). For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage [] λ , plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used. When cloning in mammalian cell systems, promoters

derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g. the retrovirus long terminal repeat; the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used. Promoters produced by recombinant DNA or synthetic techniques may also be used to provide for transcription of the inserted fluorescent protein coding sequence.

Please replace the fourth full paragraph of page 37 with the following amended paragraph:

In one aspect, FRET is used to detect the cleavage of a substrate having the donor and acceptor coupled to the substrate on opposite sides of the cleavage site. Upon cleavage of the substrate, the donor/acceptor pair physically separate, eliminating FRET. Assays involve contacting the substrate with a sample, and determining a qualitative or quantitative change in FRET. In one embodiment, the engineered fluorescent protein is used in a substrate for β -lactamase. Examples of such substrates are described in U.S. Pat. No. 5,741,657 and International Application PCT/US96/04059, filed Mar. 20, 1996. In another embodiment, an engineered fluorescent protein donor/acceptor pair are part of a fusion protein coupled by a peptide having a proteolytic cleavage site. Such tandem fluorescent proteins are described in U.S. patent application Ser. No. 08/594,575, filed Jan. 31, 1996.

Please replace the fourth full paragraph of page 40 with the following amended paragraph:

Histidine-tagged S65T GFP (R. Heim et al. Nature 373:664-665 (1995)) was overexpressed in JM109/pRSET_B in 4 l YT broth plus ampicillin at 37 °C, 450 rpm and 5 l/min air flow. The temperature was reduced to 25 °C. at A₅₉₅ = 0.3, followed by induction with 1 mM isopropylthiogalactoside for 5 h. Cell paste was stored at -80 °C. overnight, then was resuspended in 50 mM HEPES pH 7.9, 0.3 M NaCl, 5 mM 2-mercaptoethanol, 0.1 mM phenylmethyl-sulfonylfluoride (PMSF), passed once through a French press at 10,000 psi, then centrifuged at 20 K rpm for 45 min. The supernatant was applied to a Ni-NTA-agarose column (Qiagen), followed by a wash with 20 mM imidazole, then eluted with 100 mM imidazole. Green fractions were pooled and subjected to chymotryptic (Sigma) proteolysis (1:50 w/w) for 22 h at

RT. After addition of 0.5 mM PMSF, the digest was reapplied to the Ni column. N-terminal sequencing verified the presence of the correct N-terminal methionine. After dialysis against 20 mM HEPES, pH 7.5 and concentration to $A_{490}=20$, rod-shaped crystals were obtained at RT in hanging drops containing 5 μ l protein and 5 μ l well solution, 22-26% PEG 4000 (Serva), 50 mM HEPES pH 8.0-8.5, 50 mM $MgCl_2$ and 10 mM 2-mercapto-ethanol within 5 days. Crystals were 0.05 mm across and up to 1.0 mm long. The space group is $P2_1 2_1 2_1$, with $a=51.8$, $b=62.8$, $c=70.7$ Å, $Z=4$. Two crystal forms of wild-type GFP, unrelated to the present form, have been described by M. A. Perrozo, K. B. Ward, R. B. Thompson, & W. W. Ward. J. Biol. Chem. 203, 7713-7716 (1988).

Please replace the first full paragraph of page 43 with the following amended paragraph:

Although the electron density map is for the most part consistent with the proposed structure of the chromophore (D. C. Prasher et al. Gene 111:229-233 (1992); C. W. Cody et al. Biochemistry 32:1212-1218 (1993)) in the cis [Z-] configuration, with no evidence for any substantial fraction of the opposite isomer around the chromophore double bond, difference features are found at $>4\sigma$ in the final (F_o-F_c) electron density map that can be interpreted to represent either the intact, uncyclized polypeptide or a carbinolamine (inset to FIG. 2C). This suggests that a significant fraction, perhaps as much as 30% of the molecules in the crystal, have failed to undergo the final dehydration reaction. Confirmation of incomplete dehydration comes from electrospray mass spectrometry, which consistently shows that the average masses of both wild-type and S65T GFP ($31,086 \pm 4$ and $31,099.5 \pm 4$ Da, respectively) are 6-7 Da higher than predicted ($31,079$ and $31,093$ Da, respectively) for the fully matured proteins. Such a discrepancy could be explained by a 30-35% mole fraction of apoprotein or carbinolamine with 18 or 20 Da higher molecular weight. The natural abundance of ^{13}C and 2H and the finite resolution of the Hewlett-Packard 5989B electrospray mass spectrometer used to make these measurements do not permit the individual peaks to be resolved, but instead yields an average mass peak with a full width at half maximum of approximately 15 Da. The molecular weights shown include the His-tag, which has the sequence MRGSHHHHHH GMASMTGGQQM

GRDLYDDDDK DPPAEF (SEQ ID NO:5). Mutants of GFP that increase the efficiency of fluorophore maturation might yield somewhat brighter preparations. In a model for the apoprotein, the Thr⁶⁵-Tyr⁶⁶ peptide bond is approximately in the quadrature-helical conformation, while the peptide of Tyr⁶⁶-Gly⁶⁷ appears to be tipped almost perpendicular to the helix axis by its interaction with Arg⁹⁶. This further supports the speculation that Arg⁹⁶ is important in generating the conformation required for cyclization, and possibly also for promoting the attack of Gly⁶⁷ on the carbonyl carbon of Thr⁶⁵ (A. B. Cubitt et al. Trends Biochem. Sci. 20:448-455 (1995)).